

**Cell**

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The cover shows a venule (25  $\mu$ m in diameter) in the mouse mesentery, observed by intravital microscopy. Leukocytes whose motion was slowed down by rolling on the walls of the vessel are seen as refractile circles. No rolling leukocytes are observed in vessels of animals lacking P selectin. For details see the article by Mayadas et al. in this issue.

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# Leukocyte Rolling and Extravasation Are Severely Compromised in P Selectin-Deficient Mice

Tanya N. Mayadas,\*†† Robert C. Johnson,\*  
H. Ien Rayburn,† Richard O. Hynes,†§  
and Denisa D. Wagner\*

\*New England Medical Center  
and Tufts University

Department of Medicine and of Anatomy  
and Cellular Biology

Boston, Massachusetts 02111

§Howard Hughes Medical Institute

†Center for Cancer Research

Department of Biology

Massachusetts Institute of Technology

Cambridge, Massachusetts 02139

## Summary

P selectin, expressed on surfaces of activated endothelial cells and platelets, is an adhesion receptor for leukocytes. We report that P selectin-deficient mice, generated by gene targeting in embryonic stem cells, exhibit a number of defects in leukocyte behavior, including elevated numbers of circulating neutrophils, virtually total absence of leukocyte rolling in mesenteric venules, and delayed recruitment of neutrophils to the peritoneal cavity upon experimentally induced inflammation. These results clearly demonstrate a role for P selectin in leukocyte interactions with the vessel wall and in the early steps of leukocyte recruitment at sites of inflammation. These mutant mice should prove useful in deciphering the contributions of P selectin in various inflammatory responses as well as in platelet functions.

## Introduction

Emigration of circulating leukocytes from blood into surrounding tissues is a critical component of inflammation. On the other hand, excessive accumulation of leukocytes can lead to inflammatory disease. The cell adhesion events leading to arrest of circulating leukocytes and their exit from the vasculature presumably rely on the appropriate expression and function of cell surface adhesion receptors on the leukocytes and/or the endothelial cells at sites of inflammation. During the past decade, a variety of adhesion receptors have been implicated in leukocyte-endothelial interactions and are thought to play roles in recruitment, arrest, and extravasation of leukocytes (reviewed by Butcher, 1991; Carlos and Harlan, 1990; Springer, 1990).

A critical role is played by the family of  $\beta 2$  or leukocyte integrins (Arnaout, 1993; Springer, 1990). This is shown most clearly by the existence of a genetic disease, leuko-

cyte adhesion deficiency I (LADI), caused by a mutation in the  $\beta 2$  integrin gene. LADI patients have elevated levels of circulating leukocytes, but extravasation of these cells to sites of infection and inflammation is impaired. Thus, no pus forms, and the patients are subject to recurrent bacterial and fungal infections (Anderson and Springer, 1987). The  $\beta 2$  integrins promote leukocyte adhesion to activated endothelial cells but are in an inactive state and are functionally up-regulated only upon leukocyte activation. Although  $\beta 2$  integrin-mediated leukocyte adhesion is crucial, as illustrated in LADI syndrome, it does not provide the specificity for leukocyte recruitment. The effective arrest of leukocytes at a site of inflammation requires local activation of circulating leukocytes. For this to occur, the leukocytes first transiently interact with the activated endothelium, which slows their transit, thus allowing activation of their  $\beta 2$  integrins leading to arrest and extravasation. This transient interaction is manifested as rolling of the leukocytes along the vessel wall in areas of inflammation (Atherton and Born, 1972, 1973). Leukocytes from LADI patients do not roll when tested in animal models (von Andrian et al., 1993), demonstrating that rolling is not dependent on  $\beta 2$  integrins.

A variety of lines of evidence suggest that rolling is mediated by another family of adhesion receptors, the selectins (reviewed by Lasky and Rosen, 1992; Bevilacqua and Nelson, 1993). There are three known selectins, encoded by three closely linked genes clustered within 300 kb on human and mouse chromosome 1 (Watson et al., 1990). Each selectin has an N-terminal, C-type lectin domain and an adjacent epidermal growth factor-like domain connected to transmembrane and short cytoplasmic domains by a series of protein repeats related to those of complement-binding proteins. The lectin domain, and possibly also the epidermal growth factor-like domain, are essential for adhesive function and mediate the binding of selectins to specific carbohydrate structures on glycoprotein ligands (reviewed by Lasky, 1992). One of the selectins, L selectin, is constitutively expressed on neutrophils, monocytes, and many lymphocytes, whereas the other two, P selectin and E selectin, are expressed on activated endothelial cells. P selectin is also expressed on activated platelets. Although both P and E selectins are expressed on activated endothelial cells, their kinetics of expression differ markedly. Preformed P selectin is stored in endothelium-specific storage granules called Weibel-Palade bodies (Weibel and Palade, 1964; Bonfanti et al., 1989; McEver et al., 1989) and in the  $\alpha$  granules of platelets (Stenberg et al., 1985; Berman et al., 1986). It is redistributed to the cell surfaces of platelets (McEver and Martin, 1984; Hsu-Lin et al., 1984) and endothelial cells (Hattori et al., 1989) within minutes after stimulation of these two cell types with various agonists such as thrombin and calcium ionophore A23187. In contrast, E selectin is not constitutively expressed, nor is it stored. Its biosynthesis is induced by endotoxin or inflammatory cytokines with maximal surface expression 4-6 hr after stimulation (Bevilacqua

†Present address: Vascular Research Division, Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115.

qua et al., 1987). Both P and E selectins have been shown to mediate adhesion of leukocytes to activated endothelial cells (Bevilacqua et al., 1987; Geng et al., 1990) and, in the case of P selectin, activated platelets (Larsen et al., 1989).

When tested under flow conditions *in vitro*, P selectin in a lipid bilayer promotes rolling of neutrophils and is a prerequisite for activation-induced adhesion of leukocytes to the  $\beta 2$  integrin ligand intracellular adhesion molecule 1 (Lawrence and Springer, 1991). In addition, antibody inhibition experiments have implicated L selectin in mediating rolling *in vivo* (von Andrian et al., 1991; Ley et al., 1991). However, antibodies or selectin-immunoglobulin chimeras that block the functions of P or E selectin have been shown to interfere with recruitment of leukocytes at various inflammatory sites *in vivo* (Lewinsohn et al., 1987; Watson et al., 1991; Mulligan et al., 1991, 1992). So it seems likely that these two selectins are important players in leukocyte recruitment *in vivo*, presumably by promoting rolling.

To test this proposition and to investigate the biological function of P selectin, we have generated a mouse strain deficient in P selectin using the methods of homologous recombination in embryonic stem (ES) cells (reviewed by Capecchi, 1989).

## Results

### Generation of P Selectin-Deficient Mice

We isolated several mouse P selectin genomic clones and chose one containing exon 3 and exon 4 to construct a replacement vector (Thomas and Capecchi, 1987; Mansour et al., 1988). Exon 3 encodes the last 10 amino acids of the 41 amino acid signal peptide and the entire (116 amino acid) lectin domain, and exon 4 encompasses the epidermal growth factor domain. The exon-intron boundaries in this region were identical with those of the human gene (Johnston et al., 1990). We constructed our gene replacement vector so that, upon homologous recombination, this construct would replace 62 bp of intron sequences preceding exon 3 and 111 bp of the protein-encoding region (corresponding to the 10 amino acid signal peptide and 27 amino acids of the lectin domain of exon 3) with PGKneo (a neomycin expression cassette) in the opposite transcriptional orientation to P selectin (Figure 1A). We included 4.6 kb and 1.2 kb of genomic DNA upstream and downstream of the neomycin resistance cassette, respectively. To select against cells that take up DNA by random integration, viral thymidine kinase cassettes (herpes simplex virus [HSV] tk) were added to both ends of the targeting vector (Figure 1A). After transfection of D3 ES cells (derived from strain 129Sv) with the replacement vector, clones resistant to G418 and gancyclovir were screened by Southern blotting of XhoI-XbaI-digested genomic DNA; genomic sequences not included in the replacement vector were used as a probe. Three independent clones out of 184 surviving positive/negative selection (Mansour et al., 1988) were identified in which one P selectin allele had incorporated the desired mutation. The fidelity of homologous recombination was veri-

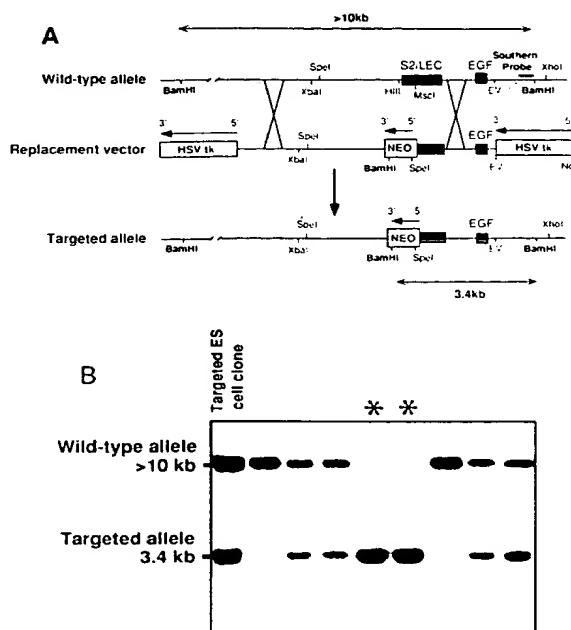


Figure 1. Design of the Replacement Vector and Southern Blot Analysis of Representative Tail Biopsies

(A) The P selectin locus (wild-type allele) and the replacement vector are shown. To construct the replacement vector, a HindIII-MscI (173 bp) fragment from the P selectin gene was deleted and replaced with a 1.7 kb neomycin gene cassette driven by a PGK promoter. The protein-encoding region deleted from exon 3 included 10 amino acids of signal peptide and 27 amino acids from the lectin domain. The 3' flanking probe used for screening ES cell clones and genotyping mice is indicated (Southern Probe). The probe detects a >10 kb BamHI fragment from the wild-type allele and a 3.4 kb BamHI fragment derived from the targeted allele, since the neomycin gene introduces a new BamHI site. EV, EcoRV; HIII, HindIII.

(B) Genomic DNA was isolated from a litter of eight mice from a heterozygous intercross. DNA was digested with BamHI, electrophoresed, and blotted. The hybridization probe was as indicated in (A). Fragments obtained from wild-type and targeted alleles are indicated. Two mice in the litter are homozygous for the mutation (asterisks). Lane 1 represents a digest of the ES cell genomic DNA. It is heterozygous for the P selectin mutation and is shown for comparison.

fied by digesting genomic DNA with other enzymes (XhoI-SpeI, XbaI-XhoI, and BamHI alone), followed by Southern blot analysis. All three digestions produced fragments of the predicted sizes (see Figure 1A), suggesting that untoward recombinations did not occur in the vicinity of the desired homologous recombination.

The clones were expanded and microinjected into C57BL/6J blastocysts to make chimeric mice. Three independent heterozygous ES cell clones gave fertile chimeras that transmitted the mutant allele to their offspring (Table 1). Heterozygous mice represented 49% of the agouti progeny of the chimeric mice, indicating that the mutant allele has no effect on viability in heterozygous mice. The heterozygous mice appeared normal in size, general health, fertility, and longevity (up to 14 months of age).

Litters resulting from intercrosses between mice hetero-

Table 1. Generation of Chimeric Mice and Germline Transmission

Chimera	Coat Color Chimerism (%)	Progeny: Agouti/Total	Germline (%)
<b>Clone 3</b>			
CM11	90	No litters	—
CM12	90	27/64	42
CM13	90	46/46	100
<b>Clone 32</b>			
CM1	90	30/32	94
CM2	80	6/24	25
CM16	70	No litters	—
CM19	90	No litters	—
<b>Clone 28</b>			
CM7	70	No litters	—
CM17	40	31/48	90
CM18	50	31/32	97

Three independent ES cell clones heterozygous for the P selectin mutation were injected into C57BL/6J blastocysts to produce chimeric animals. The percentage of coat mosaicism was scored by approximating the percentage of agouti coat color present. Germline transmission was determined by scoring the number of agouti offspring sired by chimeric males mated with C57BL/6J females.

zygous for the P selectin mutation were analyzed to determine whether or not homozygous mutant mice were viable. Litter sizes were normal, and analysis of tail biopsies revealed the presence of homozygous mutant animals (Figure 1B). Analysis of tail DNA of 340 (approximately 5-week-old) offspring, originating from all three ES cell clones, indicated that homozygous mutant mice were present at a frequency of 28%. This is close to the expected Mendelian frequency (25%), indicating that disruption of the P selectin gene did not result in embryonic lethality. The homozygous mutant mice appeared grossly normal and were comparable in body weight to wild-type animals. Nineteen wild-type and 19 homozygous mutant animals each weighed, at approximately 3 months of age,  $27.6 \pm 4.8$  g and  $27.1 \pm 4.0$  g (mean  $\pm$  SD), respectively. The animals were fertile and had normal bone structure as assayed by full-body X-rays of 3 wild-type and 3 homozygous mutant animals (data not shown). Up to 12 months of age, the general health and viability of homozygous mutant mice appeared normal.

#### Verification of the P Selectin Null Allele: RNA Analyses

To determine whether intact P selectin message was present in homozygous mutants, we performed Northern blot analysis of lung and liver RNA isolated from wild-type, heterozygous, and homozygous mutant mice. Since P selectin message in mouse tissue is present at very low levels, we treated the mice with lipopolysaccharide (LPS) for 3.5 hr prior to harvesting lung and liver for RNA isolation; LPS results in a significant increase in P selectin message in the lung and liver of mice (Sanders et al., 1992). A 3.3 kb P selectin transcript appeared in heterozygous animals at approximately half the levels present in wild-type animals, whereas homozygous mutant animals had no detectable P selectin message of the expected 3.3 kb molec-

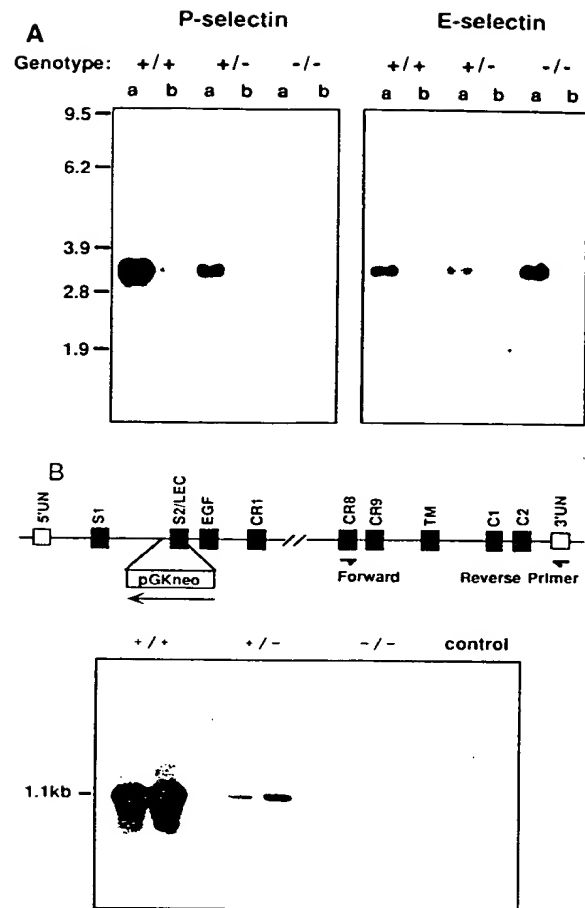


Figure 2. Analysis of Selectin Transcripts

(A) RNA samples (15  $\mu$ g) from LPS-treated mice were electrophoresed on a 1% agarose gel and subjected to Northern blot analysis with probes for mouse P selectin or rat E selectin. Samples were from lung (a) and liver (b) of wild-type (+/+), heterozygous mutant (+/-), and homozygous mutant (-/-) animals as indicated. Messages for both selectins were expressed at significantly higher levels in the lung than in the liver. RNA molecular size standards in kb are indicated to the left. In parallel, as a control for the quantity of RNA loaded, the identical loadings of RNA samples were electrophoresed and stained with ethidium bromide. 28S and 18S ribosomal bands were comparable in all the samples loaded (data not shown).

(B) Reverse transcriptase-PCR analysis of P selectin mRNA. cDNA synthesis was performed from total liver RNA of +/+, +/-, and -/- mice. In the schematic of the P selectin genomic DNA, distances between exons are arbitrary or only crudely estimated. PCR primers, as indicated, were used to amplify a fragment downstream from the insertion site of PGKneo. Amplified PCR products were electrophoresed on a 1% agarose gel and subjected to Southern blot analysis. A cDNA probe spanning the region between, and not including, the primers was used to identify P selectin-specific PCR products. The hybridizing PCR product corresponded to the expected molecular weight of 1.1 kb.

ular weight (Figure 2A). Upon longer exposure (three times that shown in Figure 2A), an RNA species of approximately 4.8 kb was observed in the mutant animals at <1% of the level of the 3.3 kb P selectin message detected in wild-type

animals. A duplicate blot was analyzed with a neomycin probe; besides the expected 0.9 kb neomycin transcript, the neomycin-derived probe also hybridized to higher molecular weight transcripts, including one of approximately 4.8 kb (data not shown). This is consistent with the possibility that the high molecular weight band observed in the homozygous animals arose from the inclusion of all or a portion of the neomycin cassette by aberrant or cryptic splicing. To confirm equal RNA loading and equivalent LPS responses in the different mice, RNA samples were also probed for E selectin mRNA. Similar levels of E selectin mRNA were observed in the lung tissue of all animals. Interestingly, liver from the same animals had very low levels (Figure 2B) of E selectin mRNA compared with lung. Differential expression after induction by LPS was also evident for P selectin message, which was much more prominent in lung than in liver (Figure 2A).

We used reverse transcriptase-polymerase chain reaction (PCR) as another approach to determine whether P selectin-specific transcripts were present at low levels in homozygous mutant animals. A 1.1 kb P selectin-specific fragment was amplified in wild-type and heterozygous animals, as expected. The same PCR product was detected at much lower levels in homozygous mutant animals (Figure 2B). We conclude that a P selectin-specific transcript of the expected 3.3 kb size cannot be detected in homozygous mutant animals using Northern blot analysis. However, a larger transcript, possibly originating from a P selectin-neomycin hybrid, is present at very low levels in tissues of homozygous mutant animals. Even if such a transcript were present, we would not expect functional P selectin to be synthesized, since our replacement vector deletes 24% of the signal peptide, the 3' acceptor site of exon 3, and 23% of the lectin domain. Deletion of a portion of the signal peptide should prevent transport of the P selectin polypeptide into the endoplasmic reticulum, and, if the P selectin molecule were to be assembled by alternate or cryptic splicing events, deletion of part of the lectin domain should render the molecule nonfunctional, since the lectin domain is required for ligand binding (reviewed by Lasky, 1992).

#### Cytofluorimetric and Biochemical Analysis of P Selectin Expression in Platelets and Lung Endothelium

To examine whether we had indeed generated a null mutation at the protein level, we studied P selectin expression in platelets and in the lung, a highly vascularized tissue. P selectin in platelets was visualized by immunofluorescence staining of resting platelets. Permeabilized platelets were stained for either P selectin or von Willebrand factor (vWf), both of which are in  $\alpha$  granules. Platelets of wild-type mice were positive for vWf (data not shown) and P selectin. On the other hand, the platelets of homozygous mutant mice gave a punctate pattern characteristic of platelet  $\alpha$  granules when stained for vWf but were negative for P selectin staining (Figure 3). We also used flow cytometry to measure levels of surface P selectin (Figure 4A). Activated platelets from wild-type animals were positive for P selectin, while the fluorescence associated with activated plate-

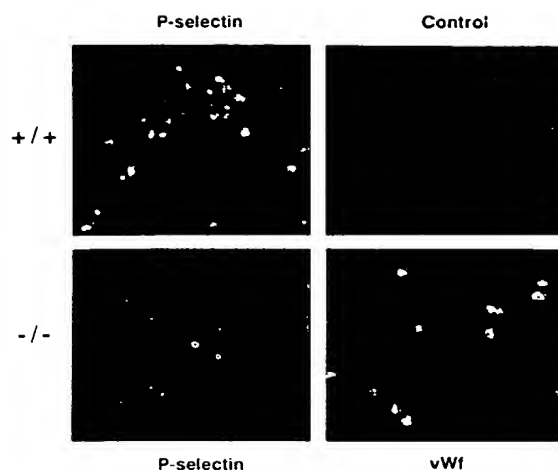


Figure 3. Immunofluorescence Analysis of Platelets

Blood smears from wild-type (+/+) and homozygous mutant (-/-) mice were prepared. They were fixed, permeabilized, and stained with either polyclonal antibody to P selectin or polyclonal antibody to vWf followed by FITC-conjugated secondary antibody; negative control was stained with secondary antibody alone. Although  $\alpha$  granules are present in both wild-type and mutant platelets, P selectin is found only in the wild type and not the mutant.

lets from homozygous mutant animals was the same as background controls. To test whether P selectin is important for granule translocation to the surface, we assayed  $\alpha$  granule release in homozygous mutant and wild-type animals. The criterion for platelet activation was the presence of vWf on the surfaces of activated platelets. vWf is not present on the surface of resting platelets but, after platelet activation, a portion of vWf released from  $\alpha$  granules binds to the surfaces of the activated platelets and can be detected with an antibody to vWf (Koutts et al., 1978). Both wild-type and homozygous mutant platelets show surface binding of vWf on thrombin activation (Figure 4B). This strongly indicates that P selectin is not required for  $\alpha$  granule release and that the absence of P selectin on the surfaces of activated platelets from homozygous mutant animals is indeed attributable to the absence of P selectin in platelets.

We examined P selectin expression in the endothelium by metabolic labeling of lung, a highly vascularized tissue. Lungs were obtained from three LPS-treated and three control animals, and each group was pooled. LPS dramatically increases P selectin message (Sanders et al., 1992) and would therefore be expected to result in increased levels of P selectin protein. Lung pieces were metabolically labeled with [ $^{35}$ S]cysteine in organ culture, and extracts were immunoprecipitated with a polyclonal antibody to vWf followed by immunoprecipitation with a polyclonal antibody to P selectin (Figure 5A). vWf was present in wild-type and homozygous mutant animals at comparable levels both before and after LPS treatment. P selectin was present in lungs of untreated wild-type animals, and its synthesis was further induced after LPS treatment. In con-

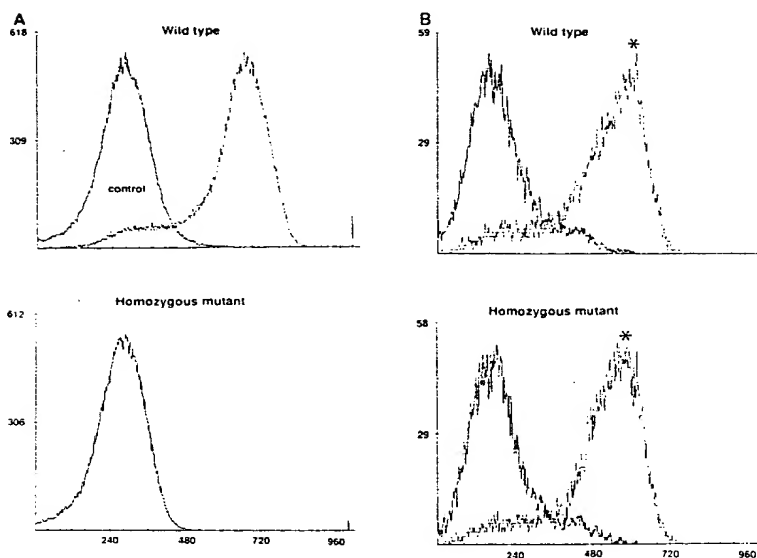


Figure 4. Flow Cytometric Analysis of Activated Platelets

(A) Platelets from wild-type and homozygous mutant mice analyzed for P selectin expression. Gel-filtered platelets were thrombin activated, fixed, and stained for P selectin. Wild-type platelets (top panel) show significant levels of surface P selectin. Platelets from a homozygous mutant mouse (bottom panel) show no staining above the background control levels seen with activated wild-type platelets stained with control rabbit antibody (control). (B) Platelets from wild-type and homozygous mutant mice analyzed for vWf expression. Resting and activated platelets were stained for surface vWf expression to assay platelet activation. The histograms for wild-type (top panel) and deficient mice (bottom panel) are displayed separately. In both cases the mean of fluorescence intensity is shifted to the same position (indicated by an asterisk) upon platelet activation, demonstrating effective release of  $\alpha$  granules in the absence of P selectin.

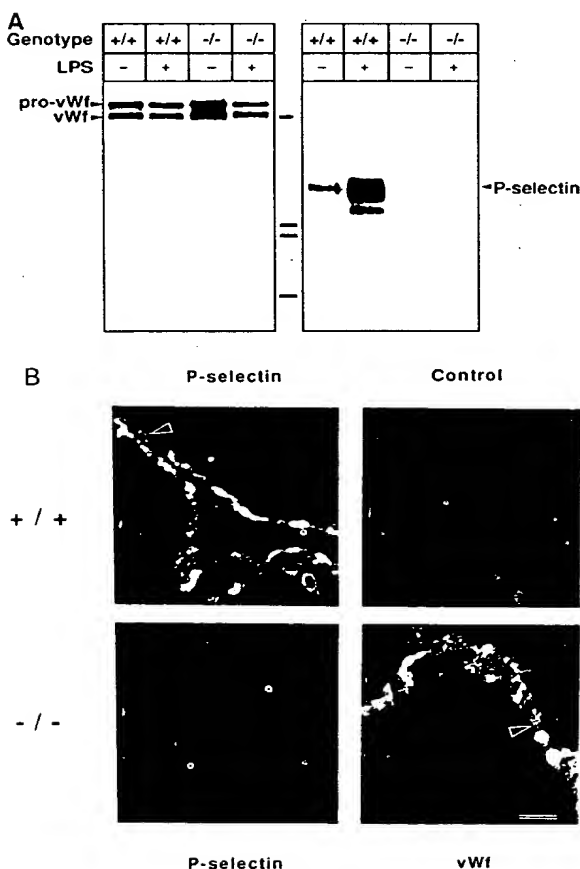


Figure 5. Analysis of P Selectin Expression in Lung Tissue  
(A) Metabolic labeling of the lung. Lungs from LPS-treated or control mice were labeled with [ $^{35}$ S]cysteine in organ culture. Detergent ex-

tracts were immunoprecipitated sequentially with antibodies to vWf (left panel) and P selectin (right panel). Reduced samples were analyzed on a 6% polyacrylamide gel. The precursor and mature forms of vWf are indicated as pro-vWf and vWf. Mouse P selectin is indicated. Molecular masses of 200, 92, 90, and 69 kd are indicated in the middle. (B) Immunofluorescence staining of lung sections. Lung sections from untreated wild-type (+/+) and homozygous mutant animals (-/-) were stained with antibodies to P selectin or vWf or control antibodies. Peribronchial venules were photographed for each panel. Although endothelial cells from mutant animals clearly contain vWf, they lack P selectin staining. Arrowheads point to granular staining pattern indicative of Weibel-Palade bodies. Bar, 20  $\mu$ m.

tracts were immunoprecipitated sequentially with antibodies to vWf (left panel) and P selectin (right panel). Reduced samples were analyzed on a 6% polyacrylamide gel. The precursor and mature forms of vWf are indicated as pro-vWf and vWf. Mouse P selectin is indicated. Molecular masses of 200, 92, 90, and 69 kd are indicated in the middle. (B) Immunofluorescence staining of lung sections. Lung sections from untreated wild-type (+/+) and homozygous mutant animals (-/-) were stained with antibodies to P selectin or vWf or control antibodies. Peribronchial venules were photographed for each panel. Although endothelial cells from mutant animals clearly contain vWf, they lack P selectin staining. Arrowheads point to granular staining pattern indicative of Weibel-Palade bodies. Bar, 20  $\mu$ m.

Table 2. Peripheral Neutrophil and Platelet Counts (Per Microliter)

Animals	Platelet Count ( $\times 10^5$ )	Total Number of Leukocytes	Neutrophil Count*
Wild-type (n = 14)	$9.8 \pm 2.5$	$7789 \pm 2455$	$843 \pm 447$
Mutant (n = 14)	$9.8 \pm 2.3$	$7813 \pm 1955$	$2018 \pm 798$

Blood samples from mice derived from all three ES cell clones were collected into a small volume of EDTA. Total leukocyte counts were determined using a Coulter counter. Platelets were counted on a hemocytometer. Blood smears were Wright's-stained, and the percentage of neutrophils present was determined by counting a total of 200 cells. The percentage of neutrophils multiplied by the total leukocyte count gave the total number of neutrophils per microliter for each animal. Data are presented as mean  $\pm$  SD.

\*  $p < 0.001$ .

### Effects of P Selectin Deficiency on Circulating Neutrophil, Leukocyte, and Platelet Counts

Peripheral leukocyte counts were determined by Coulter counts on blood samples after red blood cell lysis, and the percentages of leukocyte subpopulations were determined by differential counts on Wright's-stained blood smears. Platelet counts were determined using a hemocytometer. The results are presented in Table 2. Although the total leukocyte counts and platelet counts were not significantly different, the basal neutrophil counts in the homozygous mutant animals were, on average, 2.4-fold higher than those in the wild type. This result was confirmed by subjecting blood samples from seven wild-type

and seven homozygous animals to flow cytometry. Fluorescence-activated cell sorting density plots, which clearly separate leukocyte subpopulations by size (forward angle light scatter) versus granularity (wide angle light scatter), revealed a 2.4-fold higher granulocyte population in homozygous mutant animals compared with wild-type animals (data not shown). Therefore, by two different assays, the P selectin-deficient mice showed a 2.4-fold increase in circulating neutrophil counts (see also Figure 8). The elevation of circulating neutrophil counts in the P selectin-deficient mice suggested that there might be a defect in the interactions of neutrophils and maybe other leukocytes with the vascular endothelium in these mice.

### Leukocyte Rolling in the Microcirculation of the Mesentery

We studied leukocyte rolling by intravital microscopy of mesenteric venules (25–35  $\mu$ m in diameter) in wild-type and homozygous mutant mice. Baseline rolling was recorded during the first 10 min following surgery. The number of rolling leukocytes can vary between animals, since surgery and exteriorization of the mesentery probably cause some tissue damage, leading to variable endothelial cell stimulation. In ten wild-type mice, the leukocyte rolling index was  $10.0 \pm 2.0$  leukocytes per min. However, in eight homozygous mutant mice derived from all three mutant clones, no leukocyte rolling was observed in the venules examined (Figure 6). Although we have not examined heterozygous animals extensively, studies of two heterozygotes showed that rolling occurred within the range observed for wild-type mice.

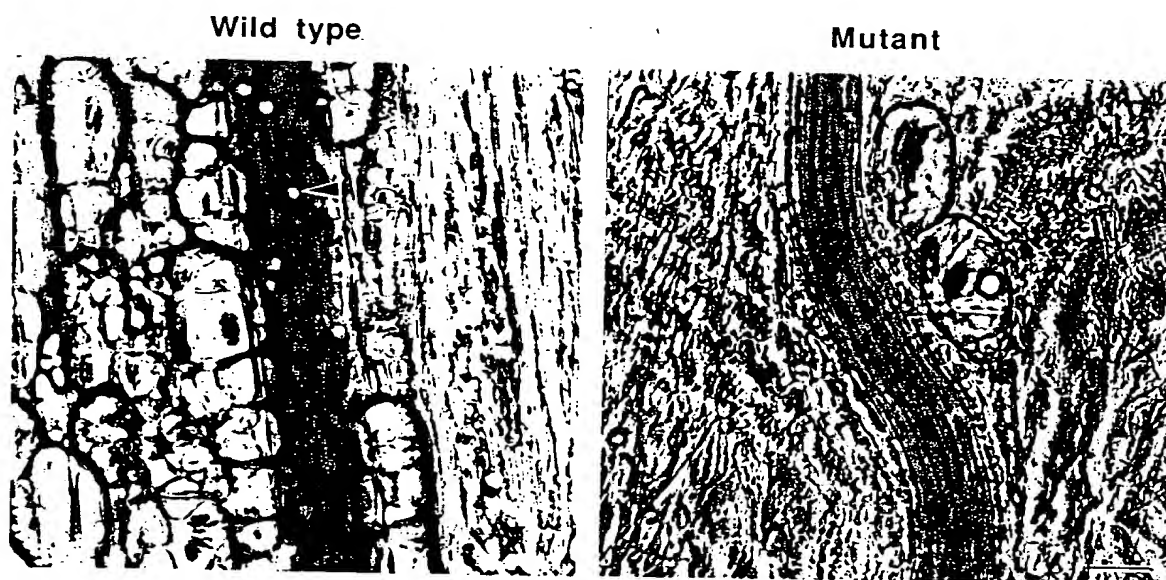


Figure 6. Basal Intravital Leukocyte Rolling

Mice were anesthetized, and a midline abdominal incision was made to expose the mesentery. Venular blood flow and diffractive, rolling leukocytes (arrowhead) were videotaped. The flux of leukocytes was quantitated by counting the number of cells passing through a perpendicular plane in 1 min. Averages were determined from the initial 10 min of filming. Photographs from one of the recordings are shown (bar, 20  $\mu$ m). Rolling leukocytes were observed in ten wild-type mice,  $10.0 \pm 2.0$  (mean  $\pm$  SEM) per minute, during a total of 53 min of recording. No leukocytes were scored as interacting with the endothelium in 38 min of recording of eight mutant mice ( $<0.03$  leukocytes per minute).



Table 3. Intravital Microscopy of Mesenteric Venules: Number of Rolling Leukocytes per Minute

Animals	Baseline	First Response	Second Response
Wild-type (n = 8)	10.5 ± 2.5	24.9 ± 7.1	17.3 ± 3.2
Mutant (n = 5)	<0.05	0.10 ± 0.06	0.04 ± 0.04

The mice were prepared as in Figure 6, and, following the 10 min baseline period, 10  $\mu$ M calcium ionophore A23187 was applied to the mesentery and venule by superfusion. Leukocyte flux determinations were made from a 20 min period following the addition of the ionophore. This period was followed by a second addition of ionophore. The table summarizes the results from 8 wild-type males and 5 P selectin-deficient males. Baseline and treatment groups were compared using the Student's *t* statistic for paired data. The *p* value for comparison of baseline rolling in the wild-type mice and initial response to ionophore is <0.05. Data are presented as mean  $\pm$  SEM.

To determine whether rolling of leukocytes could be induced in the homozygous mutant animals by enhanced activation of their endothelial cells, we added 10  $\mu$ M A23187, a calcium ionophore, to the exposed mesentery. A23187 has been shown *in vitro* to cause translocation of Weibel-Palade bodies to the endothelial cell surface within 10 min after addition (Sporn et al., 1986). After A23187 addition, leukocyte rolling was recorded for 20 min, followed by a second addition of ionophore and recording for 15 min. Addition of A23187 to the mesentery of wild-type animals resulted in about a 2-fold increase in the number of rolling leukocytes (Table 3). There was typically a gradual decline in rolling leukocytes in wild-type mice over the 20 min period following the first addition of ionophore. The second addition induced an additional, less pronounced response over baseline (Table 3). In contrast, in the P selectin-deficient mice examined, after the first A23187 addition, a total of three rolling leukocytes were observed in 2 of 5 animals examined, and after the second addition, only one rolling leukocyte was observed in 1 of the animals. We also tested hydrogen peroxide, another known *in vitro* secretagogue for Weibel-Palade bodies (Patel et al., 1991). Sixty minutes after hydrogen peroxide addition, rolling in 4 wild-type animals increased from 7.6 to 21.4 leukocytes per min (data not shown). Consistent with our data on A23187 superfusion, no leukocyte rolling was observed in 2 homozygous mutant animals either before or after hydrogen peroxide addition (data not shown). Thus, two secretagogues that result in increased rolling in wild-type mice fail to stimulate any significant rolling in the P selectin-deficient mice. The absence of increased leukocyte rolling after secretagogue treatment in P selectin-deficient mice suggests that the increase observed in wild-type animals is due to the presence of P selectin and not to nonspecific injury to the vessel wall induced by the secretagogues.

Leukocyte counts were determined from blood samples taken prior to surgery and at the conclusion of these rolling experiments. Analysis of total peripheral leukocyte and neutrophil counts revealed that homozygous mutant animals had more neutrophils than wild-type animals and comparable leukocyte counts both at the beginning and

end of the experimental procedure (data not shown). This indicates that the lack of leukocyte rolling in homozygous mutant animals cannot be attributed to systemic neutropenia or leukopenia.

#### Effect of P Selectin Deficiency on Neutrophil Influx into Inflamed Peritoneal Cavity

To determine whether neutrophils can be recruited to sites of inflammation in P selectin-deficient mice, thioglycollate, used in previous studies as an effective inducer of neutrophil-mediated inflammation (Lewinsohn et al., 1987; Jutila et al., 1989; Watson et al., 1991), was injected into the peritoneal cavity of wild-type and P selectin-deficient mice. At various time intervals, blood samples were taken to determine peripheral neutrophil counts, followed by lavage of the peritoneal cavity to examine intraperitoneal neutrophil influx (Figure 7). Seventy-five minutes after thioglycollate administration, the neutrophil influx in wild-type animals was approximately 28 times that in the homozygotes. At 135 min, wild-type animals had 6.5 times more neutrophils in the lavage than the homozygous mutant mice. At the 4 hr time point, there was significant recovery of neutrophil influx in the homozygous mutant animals, such that the difference in neutrophil influx between wild-type and homozygous mutant animals had narrowed to 2-fold (Figure 7A). To ensure that the decrease in neutrophil influx in homozygous mutant animals was not due to neutropenia, we determined total neutrophil counts in the blood samples of thioglycollate-treated animals. In both wild-type and homozygous mutant animals, the numbers of peripheral blood neutrophils increased in response to thioglycollate, and the neutrophil counts in homozygous mutant animals were elevated over those in wild-type by approximately 1.5- to 2.0-fold for all three time points (Figure 7B). This demonstrates that there is an abundance of circulating neutrophils in the P selectin-deficient animals but that the neutrophils are unable to exit the blood stream effectively at early stages of inflammation because the endothelium and/or platelets are deficient in P selectin.

#### Effects of P Selectin Mutation on E Selectin and L Selectin Expression

Since all three selectin genes are clustered within 300 kb on murine chromosome 1 in the order P selectin, L selectin, and E selectin (Watson et al., 1990), it was possible that the targeted P selectin mutation could have affected a locus control region that affects expression of all three genes. To study whether the mutation in P selectin had any effect on E selectin, we examined E selectin transcript levels in lungs of LPS-treated wild-type, heterozygous, and homozygous mutant animals. LPS has been shown to induce E selectin message. Approximately equal amounts of E selectin transcript were present in lung tissue of wild-type, heterozygous, and homozygous mutant animals. This indicates that the mutation at the P selectin locus did not affect E selectin gene transcription (Figure 2A).

To assess L selectin expression, we incubated whole blood from wild-type and homozygous mutant animals with phycoerythrin-conjugated MEL14, a rat monoclonal antibody against murine L selectin. To identify monocytes and

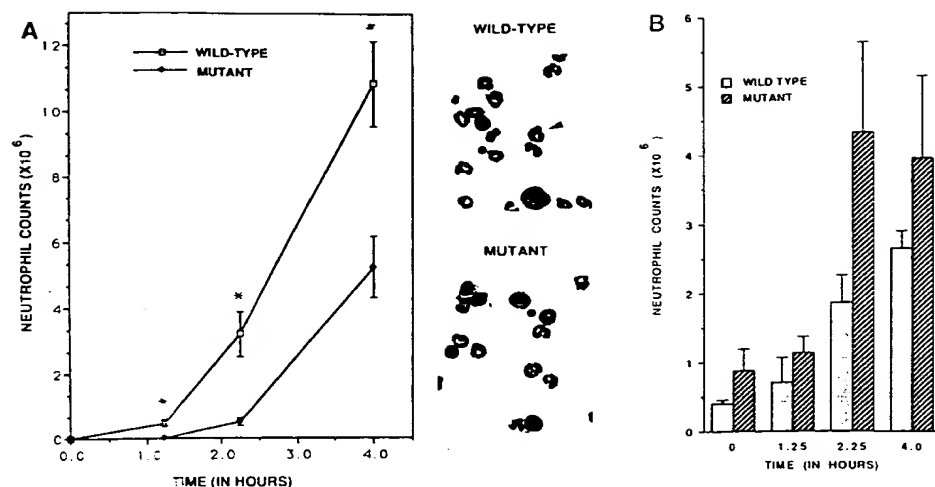


Figure 7. Peritoneal Neutrophil Influx and Peripheral Neutrophil Counts after Thioglycollate Administration

(A) At the indicated times after thioglycollate administration, total cells in the peritoneal lavage were counted, and the percentages of neutrophils in the lavage were determined. Six wild-type and six homozygous mice were used per time point, except for the zero time point (two mice of each genotype). Data are presented as mean  $\pm$  SEM. Asterisk,  $p < 0.005$ ; pound sign (#),  $p < 0.01$ . Stat-stained cytopsin preparations of the peritoneal exudate of a wild-type and a homozygous mutant animal 4 hr after thioglycollate injection are shown on the right and illustrate the appearance of polymorphonuclear neutrophils (arrowhead) present in the peritoneal lavage. Other cells in the lavage include lymphocytes, resident macrophages, mesothelial cells, eosinophils, and mast cells.

(B) Blood samples from wild-type and homozygous mutant mice were taken at the indicated time intervals following thioglycollate administration, and neutrophil counts were determined.

neutrophils positive for L selectin, we also included a monoclonal antibody to  $\alpha_M$  (subunit of the  $\beta_2$  integrin Mac-1) conjugated to fluorescein isothiocyanate (FITC). The red blood cells were lysed, and the samples were subjected to flow cytometry using two-color immunofluorescence. The mean log fluorescence for L selectin was almost identical for homozygous mutant and wild-type animals (Figure 8). In counts (mean  $\pm$  SD) on four mice of each genotype, leukocytes were  $85\% \pm 2\%$  L selectin-positive in wild-type mice and  $81\% \pm 5\%$  L selectin-positive in homozygous mutant mice, while Mac-1-positive cells (largely neutrophils and monocytes) were  $75\% \pm 6\%$  and  $72\% \pm 9\%$  L selectin-positive in wild-type and homozygous mutant mice, respectively. Parenthetically, Figure 8 also demonstrates again the elevation in Mac-1-positive cells (upper right quadrant) in the P selectin-deficient mice. To test whether A23187 treatment as used in our attempts to induce leukocyte rolling in the mutant mice might have caused shedding of L selectin from leukocytes, we added  $10 \mu\text{M}$  or  $100 \mu\text{M}$  A23187 and phycoerythrin-conjugated MEL-14 to anticoagulated whole blood for 20 min and then subjected samples to flow cytometry. The fraction of leukocytes positive for L selectin was the same with or without treatment with A23187 (data not shown). These data clearly suggest that, at the concentrations used, A23187 does not cause detectable loss of L selectin.

It follows from these results that the phenotypes discussed above for the P selectin-deficient mice are directly attributable to the mutation at the P selectin locus and not due to a disruption of the other two selectins.

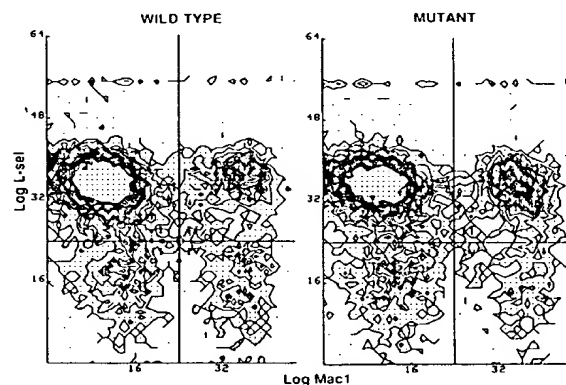


Figure 8. Cytofluorimetric Analysis of L Selectin Expression on Leukocytes

Anticoagulated whole blood collected from four wild-type and four homozygous animals was incubated with a monoclonal antibody to mouse L selectin (MEL 14) conjugated to phycoerythrin and a rat monoclonal antibody, Mac-1, conjugated to FITC. Results from one pair of animals are shown. After red blood cell lysis, cells were analyzed by two color fluorescence. The top two quadrants contain the L selectin (MEL-14)-positive cells, lymphocytes in the upper left (Mac-1-negative) quadrant and granulocytes plus monocytes (Mac-1-positive) in the upper right quadrant. Note the increase in Mac-1-positive cells in the mutant sample and the equivalent levels of L selectin expression in both Mac-1-negative and Mac-1-positive cells in wild-type and mutant samples.

## Discussion

The results reported here clearly demonstrate an important role for P selectin in leukocyte interactions with the vessel wall. The mutation we have introduced inactivates the P selectin gene, producing mice that completely lack P selectin in their platelets and endothelial cells. However, these mice continue to express L selectin and  $\beta 2$  integrin on their leukocytes (Figure 8) and are inducible for E selectin expression, just like their wild-type littermates (Figure 2A). Therefore, any defects in their inflammatory responses can be attributed to the absence of P selectin in the continued presence of other candidate adhesion receptors.

The fact that homozygous P selectin-deficient mice are viable, fertile, and of normal size and vigor demonstrates that P selectin is not required for normal development, including vasculogenesis and angiogenesis. Although P selectin expression has, to date, been reported only in platelets and endothelial cells, one could imagine roles for selectins in endothelial functions during development or in the functions of phagocytes required during tissue or bone remodeling. If P selectin does play such roles, it clearly is not absolutely required for them. P selectin does not appear to be required for platelet production and survival or for routine hemostasis. The numbers of platelets are normal, and simple tests have not revealed any obvious defects in platelet activation, aggregation, or release (Figure 4; unpublished data). Further analyses of platelet functions will be necessary to determine the roles of P selectin on activated platelets.

In contrast with the absence of obvious defects in development or hemostasis, the P selectin-deficient mice do show several alterations in leukocyte behavior. The data presented here reveal three significant differences between the mutant mice and their wild-type littermates. First, circulating neutrophil counts are elevated by an average of 2.4-fold. Second, despite this neutrophilia, leukocyte rolling in mesenteric venules is essentially ablated by the P selectin mutation. Third, there is a 1–2 hr lag in the recruitment of neutrophils to an inflammatory site. All three of these phenotypic alterations are observed in three independent lines of P selectin-deficient mice and can therefore be firmly attributed to the mutation in the P selectin gene.

The elevation of neutrophil counts is reminiscent of the situation in LAD patients. As mentioned in the Introduction, patients lacking  $\beta 2$  integrins show significant elevation (5- to 20-fold) in the number of circulating leukocytes, particularly neutrophils (Anderson and Springer, 1987). A second genetic disease, known as LADII, is caused by a defect in fucose biosynthesis. Among other consequences, this defect leads to loss of the sialyl-Lewis x carbohydrate group that is a component of the ligand for, at least, P and E selectins (reviewed by Lasky, 1992; Bevilacqua and Nelson, 1993; Varki, 1992). LADII patients also show elevated circulating neutrophil counts (Etzioni et al., 1992; Frydman et al., 1993). In all three cases, it is very likely that defects in the interactions of the neutrophils with the endothelium lead to higher numbers in the blood. Although

failures in neutrophil-endothelium adhesion are the most plausible explanation for elevated circulating neutrophil counts, other contributions, such as elevated production or release to the circulation or extended lifespan of neutrophils, cannot be ruled out at present. These possibilities can now be tested using these P selectin-deficient mice.

Spontaneous leukocyte rolling on the vessel wall was first described by Rudolf Wagner in the microvessels of frog skin in 1839. Since then, many animal models have been used to study this phenomenon. We have chosen to examine leukocyte rolling in the exteriorized mouse mesentery, since this model has been well characterized in this species (Atherton and Born, 1972). The virtual absence of leukocyte rolling in mesenteric venules of P selectin-deficient mice, even after treatment with secretagogues known to cause extensive release of Weibel-Palade bodies, is somewhat surprising. Previous studies, using antibodies, Fab fragments, or L selectin-immunoglobulin chimeras as inhibitors, have implicated L selectin in this process (von Andrian et al., 1991; Ley et al., 1991). In neither study was inhibition of leukocyte rolling complete, although it was significant (65%–90%). It is of course possible that these blocking experiments could be misleading if the antibodies or chimeras were to interfere by steric hindrance with P selectin–ligand interactions not involving L selectin. That possibility aside, the prior studies raise the question of whether L selectin function could be compromised in the P selectin-deficient mice. Several results argue against this. First, L selectin is expressed at normal levels and on normal proportions of the circulating leukocytes in the P selectin-deficient mice (Figure 8). Furthermore, the antibody we used to detect L selectin, MEL-14, is a function-blocking antibody, suggesting that the epitope crucial for L selectin function is intact. Despite the normal expression of L selectin, leukocytes did not roll on the endothelium of mesenteric venules either before or after treatment with A23187 or  $H_2O_2$ . This failure cannot be attributed to shedding of L selectin on A23187 stimulation, because, first, the deficit was observed without addition of agonists, second, A23187 did not abolish rolling in the wild-type animals (Table 3), and, third, direct tests showed that A23187 did not cause significant shedding of L selectin *in vitro*. We conclude that, if L selectin is indeed involved in leukocyte rolling, it is not sufficient by itself. Several possibilities exist. First, perhaps L and P selectins interact with each other to promote leukocyte rolling. Picker et al. (1991) suggest that L selectin on neutrophils, but not lymphocytes, is modified with sialyl-Lewis x and can thus serve as a ligand for P selectin (or E selectin). However, a recent report identifies the P selectin ligand on neutrophils as a 120 kd protein distinct from L selectin (Moore et al., 1992). A second possibility is that the absence of P selectin interferes with expression of the L selectin ligand on the endothelial surface. We were unable to test conclusively whether Weibel-Palade bodies, which may contain the L selectin ligand, can release normally in the absence of P selectin. Therefore, we cannot exclude the possibility that a necessary L selectin ligand is missing in the mutant mice as a consequence of defective Weibel-Palade body release or for other reasons. For example,

it remains possible that P selectin itself is the ligand for L selectin. However, we could show that P selectin-deficient  $\alpha$  granules do release (Figure 4B), and therefore, by analogy, it is likely that Weibel-Palade bodies can translocate to the surface in the absence of P selectin. A final possibility is that both L and P selectins are required, each interacting with its own ligand on the opposing cell to help guarantee sufficient adhesive interaction to resist shear stress. Whatever the explanation, the data show clearly that rolling requires adhesion mediated by P selectin (and perhaps also L selectin) and is not simply a consequence of the hemodynamics and rheology of the circulation (Atherton and Born, 1973; Schmid-Schonbein et al., 1980).

The final phenotype reported here, the 1–2 hr lag in recruitment of neutrophils to the peritoneal cavity after thioglycollate injection (Figure 7A), clearly implicates P selectin and, by inference, the leukocyte rolling for which it is essential in the extravasation of neutrophils from the circulation during the early stages of inflammation. Since neutrophils are the only leukocytes recruited in large numbers during this period, we cannot draw conclusions about the effect of P selectin deficiency on the recruitment of monocytes and other leukocyte subsets that contain the P selectin ligand. Similar issues concerning the relative roles of P and L selectins in this phenomenon arise here as for the leukocyte rolling discussed above, since Watson et al. (1991) have reported that antibodies to L selectin or L selectin-immunoglobulin chimeras cause partial and temporary block of neutrophil influx into the peritoneal cavity after thioglycollate. Their time courses of neutrophil extravasation are very similar to those we obtained in the P selectin-deficient mice (Figure 7A). Again, it is possible that both selectins are involved, either as complements or as obligate parallel pathways of adhesion.

The recovery of neutrophil influx at later times (Figure 7A; Watson et al., 1991) indicates that some other adhesion receptor(s) comes into play. A good candidate is E selectin, which is synthesized by endothelial cells after treatment with inflammatory cytokines and peaks at 4 hr in *in vitro* experiments (Bevilacqua et al., 1987). Antibodies to E selectin were shown to inhibit the accumulation of neutrophils in the peritoneal cavity 4 hr after intraperitoneal glycogen-induced peritonitis (Mulligan et al., 1991). Since induction of E selectin synthesis is intact in the P selectin-deficient mice (Figure 3), it would be expected to play a role in inflammation. The most plausible interpretation of our data on neutrophil influx into the inflamed peritoneal cavity is that this influx is largely dependent on P selectin at early times (<2 hr) and becomes dependent on E selectin at the later time points. This possibility is currently being examined further.

The phenotypes we have described for the P selectin-deficient mice are consistent with the rapid expression of P selectin on the surface of stimulated endothelial cells and platelets and with its role *in vitro* as an adhesion receptor for various leukocytes. Both leukocyte rolling and neutrophil extravasation are compromised in these mice. The importance of P selectin in the steady-state maintenance of the different leukocyte populations *in vivo* has not been previously appreciated. We report that P selectin defi-

ciency leads to an increase in the number of leukocytes in circulation, a phenomenon previously reported for LAD1 and LADII patients. P selectin is also expressed on activated platelets, and one might have expected obvious defects in hemostasis. Although no alterations in platelet numbers were detected, further analyses of platelet functions in these mice should help to provide insights into the functions of P selectin on activated platelets. Indeed, it is possible that the absence of P selectin on platelets contributes to the phenotype of the P selectin-deficient mice, given the potential multiple interrelationships between platelets and leukocytes in inflammation. Also somewhat unexpected is the survival of the P selectin-deficient mice up to at least 1 year of age without obvious signs of infection or disease under the conditions of standard animal husbandry. It remains to be tested how well these mice combat major experimentally induced infections. It is possible that other selectins suffice in the absence of P selectin. Surface expression of both P and E selectins can be induced by a variety of secretagogues and/or cytokines. The relative contributions of these two endothelial selectins are currently unclear but are likely to depend on the circumstances. As mentioned, L selectin likely also plays a role in many cases. Recruitment of neutrophils, monocytes, eosinophils, basophils, NK cells, and other lymphocytes to sites of inflammation is important both in normal defense mechanisms and in inflammatory diseases. All of these cells either express or can interact with one or more selectins. It will be challenging to discern which are the important players for recruitment of a given cell type in response to a given event. The availability of this strain of mice, in which one selectin is completely ablated without affecting the expression of the others, should aid greatly in deciphering the relative contributions of these adhesion receptors in various inflammatory and other biological responses.

#### Experimental Procedures

##### Isolation of Genomic P Selectin Clones

An amplified mouse genomic library constructed from the liver DNA of black agouti 129Sv mouse strain (kindly provided by Drs. H. Wu and R. Jaenisch, Whitehead Institute, Massachusetts Institute of Technology, Cambridge, Massachusetts) was screened using human P selectin cDNA (kindly provided by Drs. B. Furie and B. C. Furie, New England Medical Center, Boston, Massachusetts) as a probe. Fragments of human P selectin cDNA were prepared, and radiolabel was incorporated using random hexamer labeling techniques according to manufacturer's protocol. Prehybridization and hybridization (Sambrook et al., 1989) were performed in 40% formamide,  $5 \times$  SSC,  $5 \times$  Denhardt's, 0.1% sodium pyrophosphate, 0.5% SDS, 100  $\mu$ g/ml salmon sperm DNA. Filters were washed under low stringency ( $2 \times$  SSC, 0.1% SDS at 42°C). Several clones spanning the entire P selectin gene were isolated, and a  $\lambda$  clone with a 15 kb genomic insert was identified as containing exon 3 and exon 4 of P selectin by Southern blot analysis and sequencing. The insert was subcloned into pBlue-script II KS(+/-) (Stratagene) and extensively mapped.

##### P Selectin Homologous Recombination Constructs

One hundred eleven base pairs of the third exon and 62 bp of the preceding intron of the P selectin clone were removed by digestion with HindIII and MscI. A 1.7 kb EcoRI-HindIII pGKneo neomycin cassette that contains the promoter and 3' end of mouse phosphoglycerate kinase (pGKneo) (described by McBurney et al., 1991) was subcloned into the HindIII-MscI site in the opposite transcriptional orientation to

P selectin. The 4.6 kb HindIII-ClaI fragment and 1.2 kb MscI-EcoRV fragment of the P selectin gene were included upstream and downstream of the PGKneo cassette, respectively (Figure 1A). Two 2.7 kb HSV thymidine kinase (HSVtk) cassettes, containing phosphoglycerate kinase promoters and 3' sequences (kindly provided by Drs. M. Rudnicki and R. Jaenisch, Whitehead Institute, Massachusetts Institute of Technology), were inserted into the polylinker region of pBlue-script KS(+/-) in tandem and in the same orientation. The P selectin genomic construct containing the pGKneo gene was inserted between the two HSVtk cassettes so that the HSVtk cassettes were in the opposite transcriptional orientation to P selectin (Figure 1A). A unique NotI site in the polylinker of pBlue-script KS(+/-) of the construct was used to linearize the plasmid for transfection of embryonic stem cells.

#### Cell Lines and Tissue Culture

The ES cell line, ES-D3, derived from 129Sv 4 day blastocysts (Doetschman et al., 1985), was kindly provided by Dr. Janet Rossant (Mt. Sinai Hospital Research Institute, Toronto, Ontario). The ES cell line was cultured on mitotically inactivated G418-resistant early passage mouse fibroblasts, prepared from a transgenic strain of mice containing the neomycin resistance gene (kindly provided by Dr. R. Kemler, Max-Planck-Institut für Immunbiologie, Freiburg, Federal Republic of Germany). ES cells were grown in Dulbecco's modified Eagle's medium-HEPES (pH 7.5; Dulbecco's modified Eagle's medium purchased from Hazelton) with 15% fetal calf serum (Hazelton), 0.1 mM minimal essential medium nonessential amino acids (GIBCO BRL), 100  $\mu$ M  $\beta$ -mercaptoethanol (Fluka) and 10<sup>3</sup> U/ml leukemia inhibitory factor (ESGRO; GIBCO BRL). Cells were maintained in a 5% CO<sub>2</sub>, 95% humidity incubator.

#### Transfection and Selection

ES-D3 cells (passage 5 in our laboratory), resuspended to a density of  $2.5 \times 10^7$  per ml, were electroporated at room temperature with 25  $\mu$ g/ml of the targeting construct at 240 V, 500  $\mu$ F using a Bio-Rad GenePulser with a capacitance extender. Immediately following electroporation, cells were plated onto a monolayer of feeders. G418 (400  $\mu$ g/ml dry powder; GIBCO) and gancyclovir (2  $\mu$ M; gift from Syntex Corporation) were applied to the ES cell cultures 24 hr after plating. Cells were allowed to grow for an additional 7–8 days without passaging, and individual clones surviving G418 and gancyclovir selection were isolated and divided into two wells. Cells from one set of wells were frozen, and the other set was subjected to Southern blot analysis.

#### Southern Blot Analysis to Identify Targeted Clones and to Genotype Mice

Individual ES clones were lysed in 150 mM NaCl, 20 mM Tris (pH 7.5), 5 mM EDTA, 0.5% SDS, 0.25 mg/ml proteinase K overnight at 37°C. Samples were extracted with phenol-chloroform, and genomic DNA was isolated by ethanol precipitation. Genomic DNA (10  $\mu$ g) from each clone was digested with XhoI and XbaI. The DNA was resolved on a 1% agarose gel and transferred to nylon membrane (Zeta-Probe) using standard capillary transfer procedures. The hybridization probe was a radiolabeled 420 bp HincII-BamHI fragment of P selectin genomic DNA, which contained sequences not included in the targeting vector. Prehybridization and hybridization were carried out in 0.5 M sodium phosphate (pH 7.0), 1 mM EDTA, 7% SDS, 1% bovine serum albumin (BSA), 100  $\mu$ g/ml salmon sperm DNA at 65°C. Filters were washed twice in 40 mM sodium phosphate buffer (pH 7.0), 1% SDS, 1 mM EDTA, 0.5% BSA and twice in buffer without BSA at 65°C. Further analysis of the clones was carried out by digesting genomic DNA with other restriction enzymes: XhoI and SpeI together and BamHI alone (Figure 1A). Clones identified as being heterozygous for the desired homologous recombination event were further analyzed by Southern blot analysis, using a probe derived from the neomycin gene. To genotype mice, genomic DNA was isolated from tail biopsies using methods of Laird et al. (1991), digested with BamHI, and blotted and probed as described above.

#### Generation and Breeding of Chimeric Mice

Injections of 20 ES cells into the blastocoel cavity of 3.5 day C57BL/6J blastocysts were done according to protocols of Bradley (1987). Injected blastocysts, along with uninjected blastocysts, which served

as carriers, were implanted into the uterus of 2.5 day pseudopregnant recipients. Chimeric males were mated with C57BL/6J females at 5 weeks of age. The numbers of agouti mice versus black mice resulting from this mating were scored, and agouti mice were subsequently genotyped by DNA analysis of tail biopsies.

#### Northern Blot Analysis of P Selectin and E Selectin Expression

Total RNA from lung and liver harvested from LPS-treated mice was isolated using a guanidine thiocyanate procedure essentially as described (Sambrook et al., 1989). Between 15 and 20  $\mu$ g of total RNA was electrophoresed on a 1% agarose gel in the presence of 6% formaldehyde and subsequently transferred to Zeta-Probe membrane filter using standard capillary blotting procedures. Detection of P selectin message was accomplished by probing with a mouse P selectin cDNA fragment spanning 1.7 kb of the 3' end of P selectin (base pairs 1406 to 3075), kindly provided by Dr. D. Vestweber (Max-Planck-Institut für Immunbiologie) and previously described (Weller et al., 1992). Prehybridization and hybridization were as outlined above for Southern blot analysis. The E selectin transcript was detected using an EcoRI-SphI 1.3 kb cDNA rat E selectin fragment (Fries et al., 1993; kindly provided by Dr. T. Collins, Brigham and Women's Hospital, Boston, Massachusetts). Prehybridization and hybridization were performed as described for isolation of genomic P selectin clones. cDNA fragments used for hybridization were radiolabeled by random priming. Posthybridization filter washing was conducted under high stringency (40 mM sodium phosphate buffer [pH 7.0], 1% SDS at 65°C) for detection of P selectin transcript or reduced stringency (2  $\times$  SSC, 0.2% SDS at 42°C) for detection of E selectin transcript.

#### Reverse Transcriptase-PCR

Isolation of RNA samples from livers of LPS-treated wild-type, heterozygous, and homozygous mutant animals are described in the previous section. First-strand cDNA synthesis from 10  $\mu$ g of total RNA was performed according to manufacturer's protocols (Stratagene), using random and oligo(dT) primers. Oligonucleotide primers complementary to regions of the CR8 domain or 3' untranslated domain (Figure 2B) were incubated with one-tenth of reverse transcribed RNA mixture and subjected to 40 cycles of amplification using standard PCR protocols. PCR products were electrophoresed and transferred to nylon membrane by capillary transfer. The hybridization probe, a 1.09 kb PstI fragment of the 1.7 kb mouse P selectin fragment provided by Dr. D. Vestweber, was radiolabeled by random hexamer labeling techniques. Prehybridization, hybridization, and filter washing were carried out as described in the Southern Blot Analysis section.

#### Immunofluorescence Staining and Flow Cytometry

Blood smears were fixed in 3.7% (v/v) formaldehyde and permeabilized in 0.5% Triton X-100 in phosphate-buffered saline (PBS) without divalent cations. Cells were incubated for 30 min at 37°C with rabbit polyclonal antibody to human vWf (American Bioproducts) diluted 1:100 or with affinity purified rabbit anti-human P selectin polyclonal antibody diluted 1:100 (kindly provided by Dr. M. Berndt, Baker Medical Research Institute, Prahran, Australia), followed by a 30 min incubation with fluorescein-conjugated goat antibody to rabbit immunoglobulin G diluted 1:100 (Boehringer Mannheim).

To prepare lung sections, mice were given 0.5 ml of 3% sodium citrate (to inhibit blood coagulation) intraperitoneally and sacrificed. Lungs were immediately perfused with PBS containing 0.5 mM CaCl<sub>2</sub> and 1 mM MgCl<sub>2</sub> followed with 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) through the right ventricle. Warmed OCT was infused intratracheally, and portions of lung were embedded and frozen in OCT. Sections were permeabilized in acetone, blocked with avidin-biotin solution, and incubated with polyclonal rabbit anti-mouse P selectin antibody (kindly provided by Dr. D. Vestweber) or polyclonal vWf antibody at 37°C for 30 min. The sections were labeled with biotin-conjugated (goat anti-rabbit) (Vector) antibody followed by streptavidin-FITC (Zymed). Control sections lacked primary antibody.

Flow cytometric analysis of Mac-1 and L selectin surface expression on mouse leukocytes was performed after a 30 min incubation at room temperature of 30  $\mu$ l whole blood samples with fluorescein (FITC)-labeled rat monoclonal antibody to the mouse CD11b subunit (Boehringer-Mannheim) diluted 1:50 and phycoerythrin-conjugated rat mono-

clonal antibody to L selectin (MEL14) (Pharmingen) diluted 1:50. Negative controls were stained with FITC-conjugated rat anti-mouse immunoglobulin G alone. Erythrocytes were then lysed by adding 800  $\mu$ l of fluorescence-activated cell sorting solution (Becton-Dickinson), and FITC-conjugated monoclonal antibody and phycoerythrin-conjugated monoclonal antibody binding to leukocytes was determined on a FACScan flow cytometer (Becton-Dickinson) using two color immunofluorescence. Ten thousand cells were analyzed.

Platelets were collected after passage of platelet-rich plasma through a Sepharose 2B column (Pharmacia) equilibrated with 3.5 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 12 mM  $\text{NaHCO}_3$ , 1 mM  $\text{MgCl}_2$ , 0.1% glucose at pH 7.0. Platelets were activated by incubation with 0.5 U/ml thrombin for 20 min at room temperature. Platelets were fixed in 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) and then incubated with rabbit polyclonal antibody to human vWf (American Bioproducts) diluted 1:100, affinity purified rabbit anti-human P selectin polyclonal antibody (kindly provided by Dr. M. Berndt) diluted 1:50, or an immunoglobulin G control diluted 1:50. Platelets were then spun down, washed in PBS containing 1% BSA, and incubated in FITC-conjugated goat anti-rabbit Fab fragment (TAGO). Flow cytometry of P selectin and vWf surface expression on mouse platelets was performed. To include predominantly single platelets in the analysis of activated platelets, the window of analysis was determined using resting platelets. FITC-positive cells were determined on a FACScan flow cytometer using characteristic forward angle light scatter and one color immunofluorescence. Ten thousand cells were analyzed.

#### Immunoprecipitation from Lung Tissue and Electrophoresis of Samples

Wild-type and homozygous mutant mice were either given an intraperitoneal injection of 1.9  $\mu$ g of LPS (Sigma) per g of body weight, dissolved in 500  $\mu$ l of PBS, or left untreated (three animals for each category). At 1.5 hr after the injection, lungs were removed from untreated and LPS-treated animals, sliced into small pieces, and incubated with 1.25 mCi/ml [ $^{35}$ S]cysteine (New England Nuclear-Dupont) in Dulbecco's modified Eagle's medium-5% fetal bovine serum for 3.5 hr. Tissue was homogenized in 2% Triton X-100 in PBS supplemented with 2 mM phenylmethylsulfonyl fluoride, the sample was centrifuged at 3400 rpm for 15 min, and the pelleted debris was discarded. Prior to immunoprecipitation, the samples were incubated for 1.0 hr with gelatin-Sepharose to remove the majority of fibronectin. Subsequently, samples were sequentially incubated with polyclonal antibody to human P selectin (kindly provided by Dr. M. Berndt) bound to protein A-Sepharose and with polyclonal human vWf antibody (American Bioproducts) bound to protein A-Sepharose. Protein samples adsorbed to beads were denatured, reduced, and electrophoresed on a 6% SDS-polyacrylamide gel (Laemmli, 1970).

#### Detection of Rolling Leukocytes by Intravital Microscopy

Mice were anesthetized using tribromoethanol (0.3 mg/g), a small blood sample was obtained by an eyebleed, a midline abdominal incision was made, and the mesentery was gently exteriorized. A venule of 25–35  $\mu$ m was located for examination, and the same venule was observed for the entire procedure using a Zeiss IM35 inverted microscope (objective 32 $\times$ , 0.4 NA) connected to a SVHS video recorder (Panasonic AG-6720A, Matsushita Electric, Japan) using a CCD video camera (Hamamatsu Photonic Systems, Hamamatsu City, Japan). Exposed tissue was kept moist by periodic superfusion using PBS (without additional  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ ) warmed to 37°C. Rolling leukocytes were readily discernible as deformable, diffractive spheres (Atherton and Born, 1972, 1973) and were quantitated by counting the number of cells passing a given plane perpendicular to the vessel axis in 1 min. Baseline rolling was determined during the first 10 min after surgery by taking a minimum of four 1 min counts. A23187, a calcium ionophore, was then applied to the exposed mesentery at a concentration of 10  $\mu$ M, and four 1 min leukocyte flux determinations were made. Twenty minutes following the initial A23187 superfusion, another application of 10  $\mu$ M A23187 was made. After an additional 15 min, a blood sample was taken, and the animal was sacrificed. Blood samples taken before and at the termination of the procedure were examined for total leukocyte and neutrophil counts. For hydrogen peroxide treatment, 0.01% or 0.1% hydrogen peroxide diluted in PBS was used. Hydrogen

peroxide was added every 15 min to the exposed mesentery, and a minimum of four 1 min counts were made after each addition.

#### Thioglycollate Administration and Peritoneal Lavage

Mice were injected intraperitoneally with 1 ml of thioglycollate (kindly supplied by Dr. S. Watson, Genentech, South San Francisco, California). After various times, blood samples were taken, animals were sacrificed, and 9 ml of PBS supplemented with 0.1% BSA, 0.54 mM EDTA, and 10 U/ml heparin was injected into the peritoneal cavity. The injected wash was subsequently withdrawn while gently massaging the peritoneal wall. Total cells in the lavage were counted by Coulter counter, and cytospin preparations of the cells were stained with Wright's stain (Baxter). Cells were differentially counted to determine the percentage of neutrophils.

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